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(54) **Assay method for detecting listeria.**

(57) An assay method is provided to quickly detect the presence of Listeria strains in samples, characterized by the use of antibodies to selectively capture the peptidoglycan and teichoic acid components of the listeriae bacterial cell wall.

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	EP-A-0 303 309 (AKZO NV) * page 5, line 12 - line 46 *	1-30	G01N33/569 G01N33/68 G01N33/53
Y	US-A-4 596 769 (G.D. SCHOCKMAN ET AL.) * column 2, line 23 - line 45 *	1-30	G01N33/66
Y,D	JOURNAL OF CLINICAL MICROBIOLOGY vol. 21, no. 1, 1 January 1985, WASHINGTON DC USA pages ENZ135 - 137 K. KAMISANGO ET AL. 'Enzyme immunoassay of teichoic acids from Listeria monocytogenes.' * the whole document *	1-30	
Y,D	INFECTION vol. 16, no. SUP2, 1988, MUNCHEN BRD pages S92 - S97 F. FIEDLER. 'Biochemistry of the cell surface of Listeria strains: a locating general view.' *summary*	1-30	
A	EP-A-0 317 286 (GENE-TRAK SYSTEMS) * example 1 *	9,25,26	G01N
A	ANTIMICROBIAL AGENTS AND CHEMOTHERAPY vol. 6, no. 2, 1 August 1974, WASHINGTON DC USA pages 156 - 165 K. YOKOGAWA ET AL. 'Mutalysin, bacteriolytic agent for cariogenic streptococci: partial purification and properties.' * table 5 *	4	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	30 SEPTEMBER 1992	VAN BOHEMEN C.G.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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(54) **Assay method for detecting the presence of bacteria.**

(57) An assay method is provided to easily and visually detect the presence of organisms capable of being cultured, such as bacteria, characterized by antibody capture of the organism of interest, incubation of the captured cells to form colonies; removal of material from the colonies with a colony lift membrane; and detection of the colony material on the membrane sheet by use of labeled antibodies.

EP 0 498 920 A2

This invention relates to an assay method for quickly and easily detecting the presence of bacteria or other culturable organisms. In particular, an immunoassay method is utilized to detect the presence of viable bacteria strains in foods and other potentially contaminated samples using an assay characterized by 1) capture of specific bacterial cells with specific antibodies; (2) incubation of the captured cells to form bacteria colonies; (3) imprint of the colonies to a colony lift membrane; and (4) immunochemical detection and species identification of the colonies on the colony lift membrane.

The presence of bacterial pathogens is a well recognized cause of severe illness, so that there is an ever present need for the detection of such pathogens in both clinical specimens (i.e. blood, tissue, urine and other body extracts and fluids), agricultural specimens (such as food products) and environmental specimens (such as surfaces in food processing plants).

However, current tests for the detection of bacterial pathogens, such as in food, typically require a number of days to complete. During this period of time, between sampling and assay determination, fresh food and dairy products will enter the food chain and therefore be consumed by the public. If a test indicates the presence of pathogens, expensive product recalls may result, or, worse, before the test results are discovered an outbreak of sickness may occur.

As stated above, traditional methods to detect the presence of bacterial food pathogens require an extended period of time, basically due to the need for an enrichment/incubation period. This incubation/enrichment period is intended to allow for recovery of injured bacteria, growth of these bacteria from a background of competing microorganisms and an increase in bacterial cell numbers to more readily aid in identification. In many cases a series of two or three separate incubations is needed to isolate the target bacteria. However, such enrichment steps can actually compromise test sensitivity by killing some of the cells sought to be measured.

In the standard FDA procedure for detection of Listeria in food products (Bacteriological Analytical Manual, 6th ed., 1984; Supplement, September 1987, Chapter 29) 25 g or 25 ml of a food sample is mixed with 225 ml of enrichment broth. This sample in broth mixture is incubated for 7 days. At the end of days 1 and 7 a sample of the broth culture is streaked onto petri plates containing selective growth agar and these plates are incubated for an additional 2 days. Identification of Listeria colonies by eye confirms the presence of Listeria in the original food sample. This identification, however, is subjective, and therefore prone to misinterpretation, and this procedure requires a minimum of 7 days to confirm Listeria negative samples.

More recent methods of bacterial detection in food products have utilized immunoassays. Antibodies to an antigen present in the bacteria of interest are generally used in these methods in some form of a two site assay. That is, one antibody is immobilized and acts to capture the target bacterial antigens. This allows for separation of the target antigen from the food sample. A second antibody to this antigen (having the same or a different epitope) is labeled in some fashion such as radioactively with ^{125}I or enzymatically with horse radish peroxidase, and when added to the immobilized antibody antigen complex also becomes immobilized. Subsequent steps remove unbound labeled antibody. The label left attached is measured and usually compared against standards (positive and negative controls) to determine the presence of the target bacteria. At least one of the two antibodies used in the two site assay must be specific to the target bacteria. This type of immunoassay is known as a direct assay. Other forms, such as the competition assay are also used but tend to be less sensitive, and technically more difficult to perform.

Because of the actual sensitivity limit of these assays it remains necessary to culture the target bacteria from the food sample. In some of the newer immunoassay tests incubation times have been reduced and the number of separate incubation steps have also been reduced. The resulting tests, however, still require about 48 hours to complete.

In a generalized "rapid" immunoassay for detection of bacteria, including Listeria, 25 g or 25 ml of food sample is mixed with 225 ml of enrichment broth, as in the FDA procedure. This culture mixture is incubated for 24 hours. From this 250 ml of culture, 1 ml is removed and added to 9 ml of selective growth medium. This selective culture mixture is incubated for an additional approximately 24 hours. At this point, depending upon the actual immunoassay format, some fraction of the total 10 ml subculture (usually 0.2 ml - 1.0 ml) is tested by immunoassay for the presence of Listeria.

In summary, these existing "rapid" immunoassay procedures for bacterial detection in food samples all require at least one (usually two or more) dilution of sample (into growth medium) followed by an assay procedure which only utilizes a fraction of this final culture. The actual assay sample thus only corresponds to a small fraction of the original food sample. The bacterial culture step, or steps, must therefore overcome this dilution factor, adding to the amount of needed culture time. In addition, the utilized enrichment steps may kill the bacteria sought to be identified, producing a high

false negative rate.

These and other disadvantages of the prior art methods are overcome by the present invention which provides a fast and accurate method for the detection of viable bacteria in various possible samples.

It is therefore one object of the present invention to provide a method for quickly detecting the presence of any culturable organism, particularly bacteria, including potentially pathogenic bacteria.

It is another object of the invention to provide a method for the detection of viable bacteria which minimizes or eliminates the selective culturing steps.

It is a further object of the invention to provide a method for the detection of bacteria by which the detection can be easily made by viewing with the human eye.

These and other objects of the invention are accomplished by providing a method wherein:

(1) the bacteria cells of interest are selectively captured and removed from a sample by the use of an antibody;

(2) the captured bacteria cells are grown on a medium to form colonies;

(3) the bacteria colonies are contacted with a colony lift membrane to attach colony material to the membrane; and

(4) the presence of colony material from the colonies of the bacteria of interest is detected by use of labeled antibodies which provides visual evidence of the presence of the bacteria of interest.

As noted above, one object of the invention is to provide an assay procedure for rapid and easy identification of the presence of a particular bacteria of interest in a sample. The assay procedure is broadly applicable to the detection of any culturable organism, including bacteria, molds and yeast. Of particular importance is the detection of the presence of potentially harmful contaminants, particularly those which cannot be visually detected by eye. A broad range of contaminants can be detected by the assay, so long as the contaminant can be cultured to form colonies and antibodies can be raised against the contaminant.

In a particular preferred aspect, the assay is used to detect various bacteria, and can be utilized to detect the presence of any specific, selected bacteria of interest. The bacteria can be either pathogenic or non-pathogenic, although the invention is particularly important for detection of potentially contaminating pathogenic bacteria. Specific bacteria detectable by the assay of the invention include, for example, Listeria, Campylobacter, Escherichia coli, Salmonella, Clostridia (such as Clostridium botulinum and Clostridium perfingens), Shigella, Staphylococci (such as Stapylococcus aureus), Vibrio (such as Vibrio vulnificus, Vibrio cholerae and Vibrio parahaemolyticus), Yersinia (such as Yersinia enterocolitica and Yersinia pseudotuberculosis) Plesiomonas shigelloides, Bacilli (such as Bacillus cereus) and Aeromonas (such as Aeromonas hydrophila).

In addition, various molds can be detected, including Byssochlamys, Fusarium, Geotrichum, Penicillium and Scopulariopsis and various yeasts can be detected, such as Kluyveromyces, Pichia, Saccharomyces, Candida and Rhodotorula.

The method of the invention, therefore, generally comprises the following procedural steps:

1) The sample to be tested is, if necessary, liquified or otherwise prepared for use in the assay.

2) The liquid sample is combined with a solid matrix having immobilized thereon monoclonal or polyclonal antibodies to the selected bacteria of interest, the presence of which is to be determined by the assay. If present, the target selected bacteria cells are thereby immobilized onto the solid matrix. The solid matrix with attached immobilized bacteria is then washed to remove any remaining sample.

3) The solid matrix with the immobilized bacteria are then placed on a culture medium, and the bacteria are allowed to grow to form colonies.

4) The thus formed bacterial colonies are then contacted with a colony lift membrane, and the membrane is removed, whereby the colony material from the bacterial colonies is attached to the lift membrane.

5) The membrane is treated with a fixing agent to fix the colony material to the membrane and the membrane is then blocked to lower non-specific reactivity.

6) The membrane, having fixed thereto material from the bacterial colonies, is then treated with a labeled or unlabeled first antibody specific to the bacteria of interest to be detected, and the membrane is washed to remove any unbound first antibody.

7) If the first antibody was unlabeled, the membrane is then treated with a second antibody which is labeled and is specific for the first antibody, and the membrane is washed to remove any unbound labeled second antibody.

8) The presence of labeled first or second antibody on the membrane is then detected, specifically by means to permit visual evidence of the presence of colonies of the bacteria of interest.

As can be seen from the above method scheme, the method of the invention is particularly characterized by the use of an antibody/s to first select out bacteria from a sample. The antibody/s used for this step need not be totally specific to the bacteria of interest as subsequent selective and

specific steps combine to confer the ultimate specificity of the assay for only specific bacteria of interest. Further uniquely characterizing the method of the invention is the use of a colony lift membrane and subsequent detection steps. Using these characteristic features, the method of the invention can be importantly applied to the rapid and specific detection of viable strains of bacteria, particularly detection by a simple visual means, detectable by the human eye. The assay particularly provides a very sensitive assay detection, capable of detecting 1 colony forming unit (CFU) per 25 ml of sample.

Step 1: Liquefaction

The assay of the invention can be utilized to detect the presence of bacteria in a wide variety of samples, both solid and liquid, including food, agricultural products, environmental samples and various clinical specimens. If the sample is liquid, it can be per se subjected to the procedure of the invention, or first diluted, or concentrated by centrifugation. On the other hand, if the sample is solid, it should be first liquified (for example in water) using standard known techniques, such as by use of a blender/stomacher. The liquid or liquified sample may, if desired, be filtered through a course paper, glass or other matrix filter to remove particulates. If the sample to be tested is an environmental sample, then swabs or scrapings of the tested surface or material are mixed in a collection buffer and then treated as a liquified food sample.

Step 2: Immobilization With Antibodies

Immobilized antibodies (polyclonal or monoclonal) to target bacterial cells are used to separate the bacteria cells from the sample. In this step, one or more antibodies may be used to recognize all target strains of the bacteria genus of interest.

The antibodies used in this step recognize bacteria cell surface antigens, and these antibodies are preferably immobilized on the microspheres which can be mixed with the sample and separated from the sample mixture in some manner subsequent to capture of the target bacterial cells. In particular, microspheres of magnetic particles may be used as the immobilizing matrix. After an incubation period in which the liquid sample or liquified sample is mixed with the magnetic particles having antibodies attached, the antibody bound particles and attached bacteria are then separated from the sample by means of a magnetic field (magnetic capture) and washed to remove other potential impurities. However, in place of magnetic beads, other beads, with antibodies attached, such as latex, glass, cross-linked dextrans, agarose and polysaccharides may be used to capture the bacteria cells followed by separation from the sample mixture by centrifugation or filtration. Also filtration of the sample after bacterial cell capture over columns packed with these beads may be used to separate and concentrate the bacteria from the sample mixture. In addition, filtration of the sample over cellulose nitrate or acetate, glass fiber or nylon membranes having anti-bacteria antibodies attached and sticks or paddles coated with antibodies and stirred through samples may be used to separate the cells from the sample mixture.

As the antibodies for capture, and, as discussed later, for detection, of the bacteria, any class of antibodies can be used (including IgG and IgM) and either polyclonal antibodies or monoclonal antibodies can be used depending upon various factors, including the degree of sensitivity desired. If polyclonal antibodies are to be used, then such antibodies can be prepared according to per se known procedures. For example, procedures such as those described by Hurn, B.A. et al. (1980) in Meth. in Enzymology, Ed. Van Vanakis, H. and Langone, J., pp. 104-142, can be used.

The preparation of monoclonal antibodies is known and if monoclonal antibodies are to be used in this invention, they are prepared using the method originally authored by Milstein and Köhler and published in Nature (1975), 256, pps. 495-497. The basic process involves injecting an animal, usually a mouse, with an immunogenic substance. After suitable time for antibody production to the immunogen, the mouse is sacrificed. Cells are removed from the spleen and fused with myeloma cells. Hybridoma cells resulting from this fusion are able to reproduce in vitro, and each expresses genetic information for one specific antibody. The antibodies produced from one hybridoma fusion thus will only recognize a single antigenic determinant of the immunogen.

Cells cultured from individual hybridoma cells are screened for production of antibodies to the target antigenic determinant. Those hybridomas positive for the target antigen are further screened to identify those having any affinity. The monoclonal antibodies used in the present invention should have an affinity of at least 10^8 liters/mole. Monoclonal antibodies displaying all of these characteristics are then screened using actual assay conditions to determine if the assay condition alters the antibody binding characteristics or affinity, and to screen out those with cross reactivity to possible contaminating antigens.

In one preferred form of the invention the antibodies are immobilized on magnetic beads. This can be accomplished by procedures which are per se known, such as those described in U.S. Patent

No. 3,970,518; No. 4,018,886, No. 4,855,045 and No. 4,230,685. In one embodiment, attachment of antibodies to magnetic particles is accomplished through a Protein A intermediate. That is, Protein A is first attached to the magnetic particles and the antibodies of choice are then bound to the Protein A. The use of the Protein A intermediate greatly increases the effectiveness of capture by the attached antibodies. (Forsgren et al. (1977) J. Immunol. 99:19) Protein A attaches to the Fc portion of IgG subclass antibodies, thus extending and presenting the Fab portion of these antibodies. The resulting correct orientation of the antibodies and extension away from the particles leads to a very effective interaction between the bound antibodies and their target.

The method of attachment of Protein A to magnetic particles may proceed by any of several processes available through the scientific literature. In one such procedure, magnetic iron oxide particles of approximately 1 micrometer diameter are chemically derivatized by reaction, first with 3-aminopropyltriethoxysilane, then with glutaraldehyde. The derivatized magnetic particles are then mixed with Protein A resulting in a magnetic particle to which Protein A is covalently attached. The antibodies are then added to the Protein A magnetic particles and after a short incubation the protein A-antibody complexes form. (Weetall, H.H. (1976) Meth. in Enzymol. 44:134-148) These derivatized particles with Protein A-antibodies attached are now ready for use in bacterial cell capture.

Step 3: Growth of Bacterial Colonies

The captured and immobilized bacterial cells are placed on a medium on which the cells will grow and are incubated. Incubation is conducted for a time sufficient to form bacterial colonies visible to the eye.

Particular media for incubation depend, of course, upon the bacteria of interest to be detected. Such mediums, preferably solid, are per se known to those skilled in the art for various bacteria, as disclosed, for example, in T. Maniatis et al., Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Lab. 1982. Incubation times and conditions are also varied and per se known, depending upon the particular bacteria of interest. Generally, sufficient growth is accomplished within 6 to 24 hours.

Step 4: Colony Lift onto Membranes

After incubation of the bacteria is completed, a colony lift membrane is placed in contact with the growth medium, whereby the colonies attach and imprint colony material to the membrane. Colony lift membranes are per se known and may be comprised of, for example, nitrocellulose or nylon. The membrane is preferably cut to the size of the container or dish containing the growth medium, so that all colonies growing on the container are overlayed with the same single sheet. In this manner, the sheet/membrane acquires the same pattern of colonies that was originally contained on the growth medium. The growth medium can be maintained and utilized for multiple colony lifts from the same plate, and the lift membranes can be utilized to replicate the same colonies onto additional growth medium plates ("replica plating").

Step 5: Fixing of the Colonies to the Lift Membrane

It is then necessary to fix the colony material to the lift membrane by treatment with an appropriate fixing agent in order to ensure that the colonies adhere more firmly to the sheet. Suitable fixing treatments include placing the lift membrane in a solution of methanol and placing the membrane in a solution of the detergent sodium dodecyl sulfate with brief heating to 70 ° C.

It may also be desirable to treat the fixed colonies and membrane with per se known blocking agents to prevent non-specific reactivity of the subsequent detecting antibodies. Suitable blocking agents include, for example, caseine and BSA.

Step 6: Treatment with First Bacteria Specific Antibody

The lift membrane having fixed thereto the colony material from the bacterial colonies is next contacted with antibodies specific to he bacteria of interest. As noted above, either polyclonal or monoclonal antibodies can be utilized, but in either case have affinity for the particular bacteria to be detected. These antibodies, when contacted with the lift membrane, will adhere/bind to material from the specific target bacteria colonies, but will not bind to the other colonies.

Useful polyclonal antibodies include, for example, those from Difo poly sera raised in rabbits. These antibodies can also be specific for particular strains to be detected. For example, Listeria monocytogenes includes strains SV 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4a/b, 4b, 4c, 4d, 4e, 7, with strains SV 1/2a, 1/2b and 4b being the most common pathogenic strains. It is useful, therefore, to detect the presence in a sample of pathogenic Listeria by utilizing a polyclonal seria against Listeria serovars 1/2 and 4.

After allowing time for binding of the antibodies, the membrane sheet is washed to remove

any antibody which is not specifically bound to material from the bacteria colonies of interest.

Steps 7 and 8: Treatment of Labelled Antibody and Detection

Treatment Step 6 with a first antibody specific to the bacteria cells of interest provides the first step for selection and identification of the specific bacteria of interest. The membrane sheet is then contacted with a second antibody. Again, this antibody may be either polyclonal or monoclonal, but importantly is (a) capable of binding to the first antibody and (b) labeled in a manner to enable subsequent detection.

If the first antibody is, for example, the above-noted Difco poly sera, then the second antibody is an anti-rabbit IgG/label conjugate. If the first antibody is a monoclonal antibody derived from mouse, than the second antibody is an anti-mouse IgG/label conjugate.

Alternatively, the antibody utilized in Step 6 can itself be a labeled antibody. The assay can then proceed to detection of the label (Step 8) without the need for use of a second antibody.

With respect to labeling of the antibodies, these are labeled either directly or indirectly with labels used in other known immunoassays. Direct labels may include fluorescent, chemiluminescent, bioluminescent, radioactive, metallic, biotin or enzymatic molecules. Methods of combining these labels to antibodies or other macromolecules are well known to those in the art. Examples include the methods of Hijmans, W. et al. (1969), Clin. Exp. Immunol. 4, 457-, for fluorescein isothiocyanate, the method of Goding, J.W. (1976), J. Immunol. Meth. 13, 215-, for tetramethylrhodamine isothiocyanate, and the method of Ingrall, E. (1980), Meth. in Enzymol. 70, 419-439 for enzymes.

These detector antibodies may also be labeled indirectly. In this case the actual detection molecule is attached to a secondary antibody or other molecule with binding affinity for the anti-bacteria cell surface antibody. If a secondary antibody is used it is preferably a general antibody to a class of antibody from the animal species used to raise the anti-bacteria cell surface antibodies.

For example, the second antibody may be conjugated to an enzyme, either alkaline phosphatase or to peroxidase. To detect the label, after the membrane sheet is contacted with the second antibody and washed, the membrane sheet is immersed in a solution containing a chromogenic substrate for either alkaline phosphatase or peroxidase. A chromogenic substrate is a compound which can be cleaved by an enzyme to result in the production of some type of detectable signal which only appears when the substrate is cleaved from the base molecule. The chromogenic substrate is colorless, until it reacts with the enzyme, at which time an intensely colored product is made. Thus, material from the bacteria colonies adhered to the membrane sheet will become an intense blue/purple/black color, or brown/red while material from other colonies will remain colorless. Examples of detection molecules include fluorescent substances, such as 4-methylumbelliferyl phosphate, and chromogenic substances, such as 4-nitrophenylphosphate, 3,3',5,5'-tetramethylbenzidine and 2,2'-azino-di-[3-ethelbenz-thiazoliane sulfonate (6)]. In addition to alkaline phosphatase and peroxidase, other useful enzymes include β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-manosidase, galactoseoxidase, glucose oxidase and hexokinase.

Listeria Detection Example

The following is a specific example to show the use of the assay of the invention to particularly detect the presence of Listeria, Salmonella or E. coli.

1. A food sample is liquified and combined with magnetic beads having immobilized thereon antibodies to Listeria.
2. After an incubation time of about 30 minutes to 2 hours, the mixture is subjected to magnetic capture to remove the magnetic beads having bacterial cells immobilized thereon and the beads are washed to remove undesirable impurities and unbound material or cells.
3. The separated magnetic beads are placed on the surface of an appropriate bacterial growth medium in a Petri dish.
4. After an incubation period of time ranging from 6-24 hours, place a colony lift membrane (e.g. Bio-Rad cat. #162-0164) onto the plate of solid growth medium to obtain an imprint of the colonies on the membrane. Wait 5 minutes before peeling the membrane from the plate. The plate may be stored at 4°C, for use in future testing.
5. Place the colony lift membrane, colony side up, into a Petri dish containing 10ml methanol for 5 minutes, to kill bacteria and to fix antigens onto the membrane surface so that they will not wash off.
6. Wash the membrane with PBS (phosphate buffered saline)/0.01% merthiolate for 2-5 minutes. Remove wash fluid and repeat wash two additional times, for a total of three washes.
7. Block the membrane to prevent non-specific reactivity of the detecting antibodies by placing the membrane in 6ml of 2% non-fat dry milk/1% horse serum, PBS/0.01% merthiolate and shake

on a gyrotary shaker for 30 minutes.

8. Wash the membrane with PBS/0.05% Tween-20 for 2-5 minutes, for a total of three washes.

9. Incubate the membrane with 6 ml of first antibody, capable of binding to the bacteria of interest. The first antibody should be diluted in PBS/0.05% Tween to a good working dilution which will vary with the antibody used, and incubated with shaking of the membrane for 30 minutes.

10. Wash the membrane with PBS/0.05% Tween-20 for 2-5 minutes, for a total of two washes.

11. Incubate the membrane with 6 ml of second antibody, a goat anti-rabbit or goat anti-mouse IgG and IgM antibody conjugated to peroxidase or alkaline phosphatase. (This means the second antibodies are produced by a goat that was injected with rabbit IgG and IgM, or with mouse IgG and IgM, and the second antibodies recognize the rabbit and mouse antibodies and react with them.) These second antibodies are chemically bonded to the enzyme peroxidase, so that when a substrate for that enzyme is present, areas where the second antibody is present too will become colored. The second antibody attaches to the first antibody, which is specific for the bacteria, so that color is produced on the colony lift membrane at places where material from colonies of bacteria are present. The incubation should be in PBS/0.05% Tween 20 for 30 minutes or more, with shaking.

12. Wash the membrane with PBS/0.05% Tween-20 for 2-5 minutes, for a total of two washes.

13. Wash the membrane with water for 2-5 minutes, for a total of two washes.

14. Incubate the membrane with 6ml of fresh peroxidase or alkaline phosphatase substrate solution, with gentle shaking until brown or blue dots of colony material appear, or until the background where no colonies are present becomes pale tan or pale blue. A typical incubation requires about 5 minutes.

15. Stop the reaction by washing the membrane with water for 2-5 minutes, for a total of two washes.

16. The presence of Listeria, Salmonella or E. coli cells is then indicated by color formation on the colony material adhered to the membrane sheet.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

Claims

1. A method for detecting the presence of an organism capable of being cultured which comprises:
   a) combining a sample to be tested for the presence of a selected organism with a solid support having immobilized thereon antibodies to said selected organism, to thereby capture cells of said organism from said sample;
   b) culturing said captured organism to form colonies of said organism;
   c) contacting said colonies with a colony lift membrane whereby some colony material adheres to said membrane; and
   d) treating said membrane to provide visual evidence of the presence on said membrane of material from said colonies of said selected organism.

2. The method according to claim 1, wherein said solid support is comprised of a matrix selected from the group consisting of magnetic beads, polyacrylamide beads, agarose beads, polysaccharides, cross-linked dextrans, glass beads, latex beads, glass fiber filters, cellulose nitrate filters and nylon filters.

3. The method according to claims 1 or 2, wherein said colony lift membrane is comprised of nitrocellulose or nylon.

4. The method according to any one of claims 1 to 3, wherein after contacting with said organism colonies, said membrane is treated with a fixing agent to fix said colonies to said membrane.

5. The method according to any one of claims 1 to 4, wherein said membrane with colony material adhered thereto is contacted with detector antibodies which bind to said colony material of said selected organism and the presence of said detector antibody on said membrane is detected to provide visual evidence of the presence on said membrane of colony material from said selected organism.

6. The method according to claim 5, wherein said label is selected from fluorogenic, radioactive, chemiluminescent, bioluminescent, and enzyme substrate molecules.

7. The method according to any one of claims 1 to 6, wherein said organism is a bacteria, yeast or mold.

8. The method according to claim 7, wherein said bacteria is selected from Listeria, Campylobacter, Escherichia coli, Salmonella, Clostridia, Shigella, Staphylococci, Vibrio, Yersinia, Plesiomonas strigelloides, Bacilli and Aeromonas; said yeast is selected from Kluyveromyces, Pichia, Saccharomyces, Candida and Rhodotorula and said mold is selected from Byssochlamys, fusarium, Geotrichum, Penicillium and Scopulariopsis.

9. The method according to claim 8, wherein said selected bacteria is Listeria.

10. The method according to claim 9, wherein said selected bacteria are pathogenic strains of Listeria.

11. The method according to any one of claims 1 to 10, wherein said culturing is conducted by culturing said solid support and organism captured thereon on a solid medium.

12. A method for detecting the presence of bacteria which comprises:
a) combining a sample to be tested for the presence of a selected bacteria with a magnetic solid support having immobilized thereon first antibodies to said selected bacteria, to thereby capture cells of said selected bacteria from said sample;
b) exposing to a magnetic field said solid support having bound thereto said first antibodies and captured bacteria cells to thereby separate said magnetic solid support from said sample;
c) culturing said magnetic solid support and captured bacteria cells on a solid medium to form colonies of said bacteria;
d) contacting said colonies with a colony lift membrane whereby colony material from said colonies adheres to said membrane;
e) contacting said membrane and said colony material adhered thereto with second antibodies specific for said selected bacteria, whereby said second antibodies bind to said colony material from said colonies of said selected bacteria;
f) contacting said membrane with labeled antibodies specific for said second antibodies, whereby said labeled antibodies bind to said second antibodies bound to said colonies of said selected bacteria; and
g) treating said membrane to provide visual evidence of the presence on said membrane of said label and colonies of said selected bacteria.

13. The method according to claim 12, wherein said colony lift membrane is comprised of nitrocellulose or nylon.

14. The method according to claims 12 or 13, wherein after contacting with said bacteria colonies, said membrane is treated with a fixing agent to fix said colonies to said membrane.

15. The method according to any one of claims 12 to 14, wherein said label is selected from fluorogenic, radioactive, chemiluminescent, bioluminescent, and enzyme substrate molecules.

16. The method according to any one of claims 12 to 15, wherein said bacteria is selected from Listeria, Campylobacter, Escherichia coli, Salmonella, Clostridia, Shigella, Staphylococci, Vibrio, Yersinia, Plesiomonas strigelloides, Bacilli and Aeromonas.

17. The method according to claim 16, wherein said selected bacteria is Listeria.

18. The method according to any one of claims 12 to 17, wherein said first antibodies are polyclonal antibodies to said selected bacteria.

19. The method according to any one of claims 12 to 18, wherein said second antibodies and labeled antibodies are, or are derived from, monoclonal antibodies.

20. The method according to any one of claims 12 to 19, wherein said labeled antibody is labeled with an enzymatic molecule, which when treated, produces a color change to provide visual evidence of the presence on said membrane of said label and colonies of said selected bacteria.

21. The method according to claim 20, wherein said enzymatic molecule is peroxidase, alkaline phosphatase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-manosidase, galactose oxidase, glucoseoxidase, hexokinase.

22. The method according to claims 20 or 21, wherein said membrane is treated with a chromogenic substance for said enzymatic molecule.

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(54) **Assay method for detecting the presence of bacteria.**

(57) An assay method is provided to easily and visually detect the presence of organisms capable of being cultured, such as bacteria, characterized by antibody capture of the organism of interest, incubation of the captured cells to form colonies; removal of material from the colonies with a colony lift membrane; and detection of the colony material on the membrane sheet by use of labeled antibodies.

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EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	WO-A-8 901 162 (BIOTECHNOLOGY AUSTRALIA PTY) * the whole document *	1-21	G01N33/569 C12Q1/04 G01N33/543
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 56, no. 11, 1 November 1990, WASHINGTON DC USA pages 3478 - 3481 E. SKJERVE ET AL. 'Detection of Listeria monocytogenes in foods by immunomagnetic separation.' * page 3478, column 2, line 28 - page 3479, column 2, line 32 *	1-21	
A	EPPO BULLETIN vol. 17, 1978, WAGENINGEN NL pages 139 - 148 W.L. VAN VUURDE 'New approach in detecting phytopathogenic bacteria by combined immunoisolation and immunoidentification assays.' * the whole document *	1-21	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	14 APRIL 1993	VAN BOHEMEN C.G.

CATEGORY OF CITED DOCUMENTS

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